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Serial collection method of dog saliva: Effects of different chemical stimulants on behaviour, volume and saliva composition

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Abstract
The objective of this study was to evaluate different chemical stimulants with different flavours such as acids (citric and acetic), sweet (sucrose) and salty (sodium chloride) applied to cotton rolls and compare their effects on the volume, pH and protein concentrations of the saliva collected and the behaviour of dogs during sampling management. As an additional objective, serum cortisol concentrations of saliva samples obtained with or without citric acid and with or without previous pH adjustment were compared. Five clinically healthy were randomly assigned to one of 5 treatments with cottons with different substances: 1) control, 2) citric acid, 3) acetic acid, 4) sodium chloride, 5) sucrose. Each dog received one treatment per day, and in 5 days, all dogs were tested with the five treatments. On each day, cottons were applied to dogs at times 0, 20, 40, 60 and 80 minutes. The cottons with citric acid generated more volume than the rest of the treatments ($p<0.0001$), and sodium chloride generated more volume than the control and acetic acid ($p\leq0.03$). Cottons with citric acid generated lower pH of saliva than the rest of the treatments ($p<0.0001$). Cottons with acetic acid generated lower pH than control, sodium chloride and sucrose ($p<0.0001$). There were no differences in cortisol concentrations between the control samples and those obtained with citric acid, nor between these same samples with and without pH adjusted with buffer. The concentration of proteins in saliva and excitement degree did not change with treatment. Citric acid was more palatable than the rest of the treatments ($p<0.0001$). Sodium chloride and sucrose were more palatable than control ($p<0.05$). In conclusion, citric acid was the chemical stimulant that generated greater volume of saliva and greater palatability in dogs. Although the pH of the saliva obtained with citric acid was clearly acidic, its acidic pH did not affect the determination of cortisol by chemiluminescence or RIA. Sodium chloride and sucrose allowed to obtain high volumes of saliva and were more palatable than the control, which can be other interesting options to obtain saliva in case of not being able to use citric acid.
Keywords: Canine, Citric, Cortisol, Palatability, Sucrose.

Introduction

Saliva as a biological sample for the determination of biomarkers in veterinary medicine is being used more frequently. Most of the works on biomarkers in dog saliva were focused on cortisol determination (Vincent and Michell, 1992; Beerda *et al*., 1999; Kobelt *et al*., 2003; Dreschel and Granger, 2009; Bennett and Hayssen, 2010; Cobb *et al*., 2016). In addition to the determination of cortisol, other molecules such as hormones; oxytocin and vasopressin (MacLean *et al*., 2018), immunoglobulins and albumin (German *et al*., 1998; Clemente *et al*., 2010), acute-phase proteins (Parra *et al*., 2005), enzymes (Tvarijonaviciute *et al*., 2017) and drug monitoring in pharmacological studies (Watanabe *et al*., 1981, 1985) have been made from dog saliva samples.

In comparison with blood samples, saliva sample has the advantage of being non-invasive and therefore has important benefits on animal welfare. Besides, Mitsouras and Faulhaber (2009) concluded "that saliva presents a non-invasive alternative source of high quantities of canine genomic DNA suitable for genotyping studies" and Diverio *et al.* (2015) showed "that saliva is useful for assessing metabolism- and oxidative stress-related genes without the need for restraint". Based on the information presented, it is highlighted the advantages of using saliva as a non-invasive method and the multiplicity of its uses and applications in dogs.

Although saliva as a biological sample has advantages of being non-invasive, it has the disadvantage to present great variability in the volume obtained, which can be in the range of 0 to 1.5 mL (Dreschel and Granger, 2009). In addition, Srithunyarat *et al*. (2018) reported that saliva volume collected without stimulants form some dogs were insufficient for laboratory analysis. In

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dogs of small or very small size or in those that need to be fasting for several hours, as it can happen for example in pharmacology studies (Thombre, 2004), obtaining and assuring minimum volumes of saliva is a challenge.

Several methods have been tested to collect saliva samples, with different materials (cotton: such as cotton swab, synthetic swab, cotton bud; as well as hydrocellulose and polymer) and different stimulants (citric acid, food prospect, beef flavor, chewing) (Kobelt *et al*., 2003; Dreschel and Granger, 2009; Lensen *et al.*, 2015; Cobb *et al.*, 2016). Among the chemical stimulants used for production of canine saliva, citric acid is the most reported in scientific works (Kobelt *et al.*, 2003; Dreschel and Granger, 2009; Lensen *et al*., 2015; Cobb *et al*., 2016). However, according to our knowledge, there are no works that have evaluated the volume of saliva obtained with citric acid or with others chemical stimulants in samples collected in serial form over time.

On the other hand, a study conducted *in vitro* by Dreschel and Granger (2009) reported that the addition of citric acid to saliva samples decreased the pH of the sample and affected the measurement of cortisol. In this sense, the use of other non-acidic substances with different flavours such as sweet or salty could have benefits in obtaining samples of saliva without affecting its pH. Likewise, substances with different flavour may affect the palatability (Thombre, 2004; Aldrich and Koppel, 2015). In studies on food or pharmacological formulations in dogs, the palatability is mainly based on consumption and non-consumption tests, and within the consumption tests in acceptance or preference tests (Thombre, 2004; Payne-Johnson *et al.*, 2007; Verbrugghe *et al.*, 2012; Aldrich and Koppel, 2015). However, it has not been reported how different substances can affect palatability during saliva sampling in dogs. Since in rodents palatability has been evaluated based on the frequency of licks (Davis and Smith, 1992; Dwyer, 2012; Lin *et al*., 2014; Johnson, 2018), it is possible that the evaluation of this behaviour is also helpful to evaluate palatability during saliva sampling in dogs. In this sense, it can be expected that substances that are more palatable than others allow to have advantages in the volume of saliva obtained, since the dogs could lick the cotton rolls spontaneously and load them with more saliva. Therefore, testing different substances with different flavours could also be a benefit from the point of view of animal welfare during the sampling of saliva.

Therefore, the objective of this study was to evaluate different chemical stimulants with different flavours such as acids (citric and acetic), sweet (sucrose) and salty (sodium chloride) applied in cotton rolls and compare their effects on the volume, pH and protein concentrations of the saliva collected and the behaviour of the dogs during sampling management. As an additional objective, serum cortisol concentrations of saliva samples obtained with or without citric acid and with or without previous pH adjustment were compared.

Materials and Methods

All the experimental procedures were approved by the Comisión Honoraria de Experimentación Animal (CHEA) of the Facultad de Veterinaria, Universidad de la República (PI No 639/2017).

Location, animals and management

The study was conducted at the Laboratory of Clínica Semiológica of the Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay. Five clinically healthy dogs (three females and two males), one of Cocker breed and four mixed breed ranging in age from 1.5 to 8 years and with body weights from 11,4 to 32 Kg were used in this study. All dogs were kept in individual locations (18-22°C) from before and during the experimental period. The dogs were fed standard dry maintenance diets for adult (Labrador diet, Royal Canin). Before starting the saliva sampling procedures the dogs were fasting for more than 8 hours.

Cotton preparation with different substances

All substances (sucrose, NaCl, citric and acetic acid) were prepared at 5 % (Cronin *et al*., 2003; Kobelt *et al*., 2003), and 1.5 mL of each solution was added to two thirds each cotton dental roll of 4 cm long x 1.0 cm wide (Fig. 1A). These cotton rolls, soaked in different substances were dried in an oven at 60°C for 5 h, in a way similar to that reported by Kobelt *et al.* (2003).

Sampling procedures

The five dogs were randomly assigned to one of 5 treatments with cotton rolls with different substances: 1) control (cotton without any substance), 2) citric acid, 3) acetic acid, 4) sodium chloride, 5) sucrose. Each dog received one treatment per day, and in 5 days, all dogs were tested with the five treatments. On each day (between 9:00 and 11:00 am), cottons were applied to dogs at times 0, 20, 40, 60 and 80 minutes, of which time 0 and 20 minutes were used as baseline for each animal per day and for each treatment, and therefore those cottons do not have chemical substances. The substances were tested at times 40, 60 and 80 minutes after sampling began.

The sampling procedure consisted in picking a cotton roll by one of the ends with a mouse-tooth hemostat (Fig. 1A), locating the cotton about 5 or 7 cm away from the dog's snout for 3 sec, in order to present the cotton roll and letting it smell it (Fig. 1B). If after smelling the cotton roll the dogs lick the same, the cotton roll is kept close to the mouth to facilitate the licking (Fig. 1.D). If after smelling the cotton the dogs do not lick the same, the cotton is placed in the mouth of the dog, so that the same has contact with the oral

cavity (tongue and palate) and the vestibular cavity (between teeth and cheeks) (Fig. 1C). Then allow the dog to bite the cotton roll at least once, and then again to place the cotton roll in the oral and vestibular cavity until it is imbibed with saliva. After one minute, the cotton roll was placed on a nylon mesh (2 mm diameter pores), which was located inside a 15 mL falcon tube (Fig. 1E).

Immediately after obtaining the sample and located in the falcon tubes, the same is placed on ice. The procedure lasted one minute per cotton roll, and done in duplicate at each of the times mentioned above. The tubes were centrifuged at 3500 rpm for 15 min at 4°C and saliva samples stored at -20°C.

Determination of volume, pH and protein concentration in saliva

After the tubes were centrifuged, the volume of saliva obtained from each cotton roll was measured by a micropipette and the results are expressed as the total volume in mL (sum of the volume obtained in each pair of cotton rolls in each sampling time). The pH of saliva was tested using pH indicator strips (pH 0-14) and total protein concentrations in saliva were determined by Lowry method (Lowry *et al.*, 1951).

Determination of cortisol concentrations

Samples obtained from cottons without substances (control, n=6) and samples obtained with cottons with citric acid (n=6) were used for the determination of cortisol. Cortisol concentrations were determined in the same samples (control and with citric acid) at their original pH and at one pH adjusted between 8-9 using Tris buffer and a concentrated solution of NaOH. Cortisol saliva concentrations were determined by a competitive chemiluminescent enzyme immunoassay (Immulite® 1000 analyzer using a Siemens Cortisol kit; Los Angeles, USA). To confirm if citric acid affects the determination of the concentration of cortisol in saliva, the same samples obtained with and without citric acid (previously analyzed by chemiluminescence) were analyzed by radioimmunoassay (RIA) with a kit HTRFR-CORT-CT2 (Cisbio Bioassays, CIS Bio International, Bedford, USA). The intra-assay and inter-assay coefficient of variation were less than 10 %.

Dog behaviour

The dogs were filmed to record their behaviour during handling sampling. The excitation of the dogs during handling of saliva sampling was evaluated on a subjective scale from of 1 (very calm) to 5 (very excited), according to Dreschel and Grager (2009). To evaluate the palatability, we generated a scale of 1 to 4, being 1 non-palatable (the dogs do not lick the cotton roll and try to avoid it) and 4 very palatable (the dogs after sniffing the cotton roll or after introducing the cotton in the mouth they lick it spontaneously and with high frequency).

Statistical analysis

Volume, pH, saliva protein concentrations and behaviours were analysed by ANOVA with the mixed model of SAS University Edition. The model included the treatment (control, citric acid, acetic acid, sodium chloride and sucrose), the time, and the interaction between treatment and time as fixed effects, and the dog into each treatment as a random effect. The initial data (time 0) were included as covariates in the model. The concentrations of cortisol and pH in control samples and in samples obtained with citric acid (with and without pH control) were compared with one-way ANOVA. Data are presented as mean ± SEM.

Results

Volume, pH and protein concentration in saliva

The range of saliva volume obtained in all samples was between 0 and 1.310 mL. There was an effect of the treatment ($p<0.0001$), time ($p<0.0001$) and interaction between treatment and time ($p<0.0001$) in the volume of saliva obtained. The cotton rolls with citric acid generated more volume than the rest of the treatments ($p<0.0001$, Fig. 2A). After citric acid, the treatment that generated the most volume of saliva was sodium chloride, which was greater than the control ($p=0.02$) and acetic acid ($p=0.03$), but not different from the sucrose treatment (Fig. 2A). There was no difference in the volume of saliva obtained between the treatment with acetic and control. The volume of saliva obtained with citric acid increased from 20 min to 40 min ($p<0.0001$), and remained at high levels until 80 min (Fig. 2A). The volume of saliva obtained with sodium chloride increased from 20 min to 40 min ($p=0.03$), and remained at high levels until 80 min (Fig. 2A). The volume of saliva obtained with sucrose increased from 20 min to 40 min ($p=0.03$), and then decreased from 40 min to 80 min ($p=0.04$) (Fig. 2A). The volume of saliva obtained in the control and treatment with acetic acid did not change with time (Fig. 2A). There was an effect of the treatment ($p<0.0001$), time ($p<0.0001$) and interaction between treatment and time ($p<0.0001$) in the pH of saliva. The cotton rolls with citric acid generated lower pH of saliva than the rest of the treatments ($p<0.0001$, Fig. 2B). The cotton rolls with acetic acid generated lower pH than control, sodium chloride and sucrose ($p<0.0001$, Fig. 2B). There were no differences in the pH of saliva between the control, sodium chloride and sucrose (Fig. 2B). With both acids (citric and acetic), the pH decreased from 20 min to 40 min ($p<0.0001$), and remained at low values until 80 min, while the pH of the rest did not change with time (Fig. 2B).

The concentration of proteins in saliva (mg/mL) did not change with treatment (control: 2.7±0.5, citric acid: 1.6±0.6, sodium chloride: 1.5±0.5, acetic acid: 1.6±0.7, sucrose: 2.1±0.6) or time, nor was there interaction between treatment and time.

Fig. 1. Images of the saliva sampling used: A: hemostatic clamp with mouse-tooth holding the cotton roll at one end, B: presenting and locating the cotton roll about 5 or 7 cm away from the dog's snou, C: cotton roll placed in the mouth of the dog, so that the same has contact with vestibular cavity (between teeth and cheeks), D: dog licking the cotton roll, E: cotton roll placed on a nylon mesh located inside and up to half of 15 mL falcon tube.

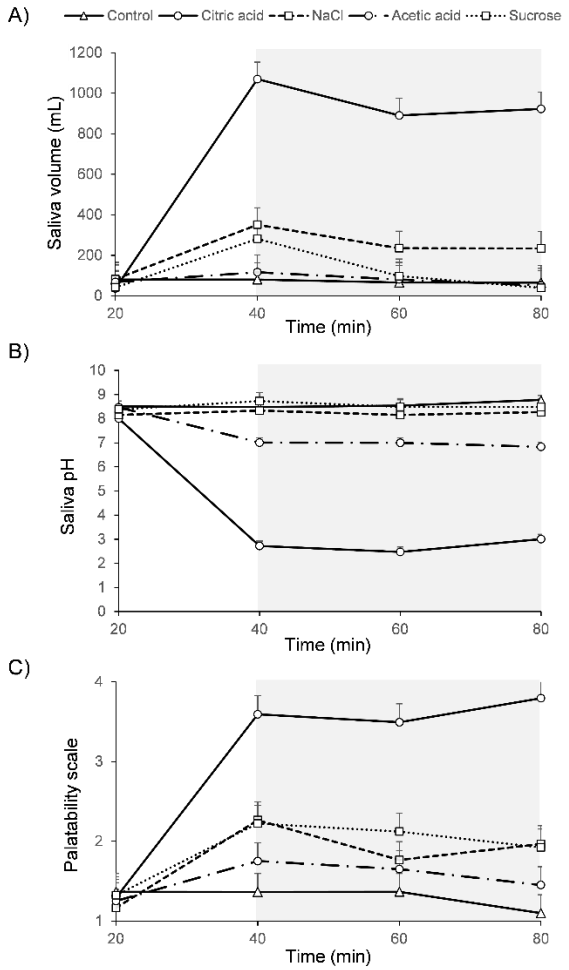

Fig. 2. Total volume (mL) of saliva (A), pH of saliva (B) and palatability (C) in each of the sampling times (20, 40, 60 and 80 min). The time 20 min corresponds to the time in which the used cottons did not have substances, while the times represented under the shaded area (40, 60 and 80 min) correspond to the times in which each substance was applied in the respective treatments (Data presented as mean±SEM).

Cortisol concentrations

Figure 3 shows the concentrations of cortisol determined by chemiluminescence and the corresponding pH of the samples obtained with citric acid and control and with or without pH adjustment with buffer. There were no differences in cortisol concentrations between the control samples and those obtained with citric acid, nor between these same samples with and without pH adjusted with buffer (Fig. 3). In these samples, the saliva pH was lower with citric acid than with control and with pH adjustment ($p<0.0001$, Fig. 3). In addition, there was no difference in the concentrations of cortisol measured by RIA between control samples (0.13±0.04 µg/dL) and those obtained with citric acid (0.16±0.02 µg/dL).

Dog behaviour

The excitement degree did not change with treatment (control: 2.1±0.2, citric acid: 1.9±0.2, sodium chloride: 1.9±0.2, acetic acid: 1.7±0.2, sucrose: 2.1±0.2) or time, nor was there interaction between treatment and time. The palatability was affected for the treatment ($p<0.0001$), time ($p<0.0001$) and interaction between treatment and time ($p<0.0001$). Citric acid was more palatable than the rest of the treatments ($p<0.0001$, Fig. 2C). Sodium chloride and sucrose were more palatable than control ($p<0.05$), but they were no different of acetic acid treatment (Fig. 2C). The palatability with citric acid, sodium chloride and sucrose increased from 20 min to 40 min ($p<0.01$) and all of them remained at high levels until 80 min (Fig. 2C). The palatability in the control and in acetic acid treatment did not change with time (Fig. 2C).

Discussion

In this study, we describe a method to obtain dog saliva repeatedly over time and with different chemical stimulants in an easy and economical way. In general, the range of saliva volume, pH, total protein and cortisol concentrations obtained in this study was within the range previously reported by others studies (Watanabe *et al.*, 1981, 1985; Dreschel and Granger, 2009; Lensen *et al.*, 2015; Cobb *et al.*, 2016).

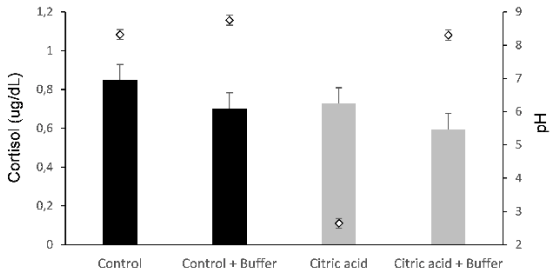

Fig. 3. Saliva cortisol concentrations (mean±SEM) in samples control (obtained without substances, black bars) and with citric acid (gray bars), both with or without addition of buffer. The pH of saliva (mean±SEM) in each sample (control and with citric acid, and with or without addition of buffer) it is shown with white rhombuses inserted in the figure.

The degree of excitation during the sampling was not affected by the treatment and dogs were relatively calm, according to Dreschel and Granger (2009). Therefore, although saliva samples were obtained serially over time, the method described did not affect the excitement or nervousness behaviour of these animals.

Of the chemical stimulants tested, citric acid was the one that obtained the highest volume of saliva, and whose volume remained high in the successive times. The treatment with sodium chloride and sucrose also allowed to obtain greater volumes of saliva than the control. These results are important to take into account for those researchers who need to obtain high volumes of saliva or at least to ensure minimum volumes of saliva (and not discard samples) for experimental studies with dogs. Our results highlight the usefulness of citric acid as an important stimulant of saliva flow in dogs, as reported in several studies (Kobelt *et al*., 2003; Dreschel and Granger, 2009; Lensen *et al*., 2015; Cobb *et al*., 2016).

To the best of our knowledge, this is the first report showing a way to evaluate the palatability of different substances applied to cotton rolls during saliva sampling in dogs. Citric acid was more palatable than rest of treatments, which generates another advantage not only in obtaining greater volume of sample, but also in its positive effect on the behaviour and welfare of the dog during sampling. This result reinforces the benefits of using citric acid as a stimulant of the flow of saliva in dogs. Although was suggested that dogs prefer sweet substances and cats acid substances (Grace and Russek, 1969; Ferrell, 1984; Bradshaw, 1991; Thombre, 2004), under the conditions carried out in this work, citric acid was more palatable than sucrose. In addition, not all acidic substances have the same flavour. Since the palatability was different in samples obtained with citric acid than in those obtained with acetic acid, it is suggested that the palatability is not directly associated with the pH of the sample, but directly with the flavour of the substance itself. The treatment with sodium chloride and sucrose were also more palatable than the control. The fact that sucrose increase palatability is in agreement with other studies conducted with water or food in dogs (Grace and Russek, 1969; Ferrell, 1984; Bradshaw, 1991; Félix *et al.*, 2012). Besides, it has been reported also that the use of salts (eg, NaCL) increases the preference for food in dogs (Delaney, 2006; Chandler, 2008). Based on these results it is suggested that in the case of not being able to use citric acid due to the decrease in the pH of the saliva sample, the use of sodium chloride or sucrose may be other good options. According to our knowledge, there are no studies that have evaluated sodium chloride or sucrose to obtain saliva in the way described in this study. Considering that with the proportions used (5 %) was obtained less volume than that used with citric acid, it would be interesting to be able to test the use of sodium chloride and sucrose at greater percentages. Moreover, it is also important to consider that many factors depending from dog (such as sex, age, animal's oral health), from pet owner and environment (temperature, noise, and other elements in the testing room) could influence the test (Delaney, 2006; Verbrugghe *et al*., 2012). Although future work is necessary to reinforce these findings, evaluating the palatability of the form described in this paper could open new ways of evaluating different substances not only for obtaining saliva, but also for testing substances individually or in conjunction with other in the food industry and in pharmacological formulations. On the other hand, samples obtained with citric acid decreased the pH of saliva. This result was different form that reported by Dreschel and Granger (2009), who observed that pH of saliva obtained with citric acid was equal to or greater than obtained with control samples, suggesting that the saliva buffer could avoid the pH changes generated by the citric acid. Although in our work the use of citric acid decreased the pH of saliva, we did not observe differences in the concentration of cortisol between samples with and without citric acid nor between the same samples obtained with citric acid with and without adjusted pH. This result also differs from that obtained by Dreschel and Granger (2009), who reported *in vitro* studies that changes in the pH of saliva generate changes in the concentration of cortisol. The differences between our work and that of Dreschel and Granger (2009) in relation to the pH of the sample and the cortisol concentrations can be explained by the use of different techniques. We used chemiluminescence and RIA, while Drerschel and Granger (2009) used ELISA. Although future works are needed to confirm these results, possibly not all cortisol determination techniques have the same interference due to changes in pH of the samples.

In conclusion, citric acid was the chemical stimulant that generated greater volume of saliva and greater palatability in dogs. Although the pH of the saliva obtained with citric acid was clearly acidic and lower than the rest of the substances tested, the acidic pH did not affect the determination of cortisol by chemiluminescence or RIA. Sodium chloride and sucrose allowed to obtain high volumes of saliva and were more palatable than the control, which can be other interesting options to obtain saliva in case of not being able to use citric acid.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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